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Award Number: W81XWH-07-1-0471

TITLE: Targeted Lymphoma Cell Death by Novel Signal Transduction Modifications

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REPORT DATE: July 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
14-07-2010	Annual	15 JUN 2009 - 14 JUN 2010

4. TITLE AND SUBTITLE
Targeted Lymphoma Cell Death by Novel Signal Transduction Modifications

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0471

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California Davis
Davis, CA 95616

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The proposed research set to; 1) create and characterize CD22-binding peptides that initiate signal transduction and apoptosis in non-Hodgkin's lymphoma (NHL), 2) optimize CD22-mediated signal transduction and lymphomacidal properties of ligand blocking anti-CD22 monoclonal antibodies (mAbs) and peptides with CD22-specific phosphatase inhibition and 3) correlate mAb-mediated and anti-CD22 peptide-mediated in vivo physiologic changes, efficacy, and tumor targeting using advanced immuno-positron emission topography (i-PET) and FDG-PET imaging technology. Since funding we have identified five peptides that are based on CDR's of anti-CD22 mAbs. Peptide 5 has been characterized and described in the annual report for year 1 and 2. Within year 2 & 3 we have identified several other peptides that are more effective that peptide 5 and we have begun to characterize their specificity, signaling, cytotoxic and apoptotic potential. In addition we have initiated studies to identify CD22 binding peptides with greater affinity and specificity.

15. SUBJECT TERMS
CD22, lymphoma, peptides

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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I. Introduction

CD22 is a B-lymphocyte-specific glycoprotein that can function as an adhesion molecule capable of binding multiple hematopoietic cell types; it can also transduce signals to the cell interior. Our studies have begun to dissect the CD22 signaling cascade at the biochemical level. We identified anti-CD22 monoclonal antibodies (mAb) that bind the two NH_2-terminal immunoglobulin domains of CD22; these mAb specifically block the interaction of CD22 with its ligand. CD22-blocking mAb are highly effective at inducing proliferation of primary B-cells but the CD22 blocking mAb produce apoptotic responses in neoplastic B-cells (1-3). Our lab and others have demonstrated that ligand blocking mAbs have *distinct* functional properties. We identified anti-CD22 mAbs that are unique and functionally distinguishable from other anti-B-cell, and even other anti-CD22 mAb (4-6). In fact, the NCI has approved and funded the humanization of the anti-CD22 blocking mAb, HB22.7 through the Rapid Access Intervention Drug (RAID) Program. Humanized HB22.7 could become an exciting new therapy for patients with CD22-positive non-Hodgkin's lymphoma (NHL), much as rituximab (Rituxan) is an option to patients with CD20-positive NHL.

By sequencing the heavy and light chain variable regions of five anti-CD22 blocking mAbs, we identified highly conserved complementary determining regions (CDRs) that bind CD22, and initiate CD22-mediated signal transduction. Anti-CD22 peptides were created based on the CDRs. We hypothesize that these unique peptides derived from the anti-CD22 mAb CDRs can be effective therapy against NHL and autoimmune disease. Furthermore, we hypothesize that the peptides that initiate signaling and enter B-cell NHL will be the cornerstone for development of a CD22-based drug delivery system. These novel, new anti-CD22 peptides may be even more effective than their parent mAbs, and the "next step" toward a new generation of effective anti-NHL drugs.

In addition, our understanding of CD22-mediated signal transduction allows us to demonstrate that phosphatase inhibition can lead to enhanced CD22-mediated signals, apoptosis, and lymphomacidal effects in human NHL xenografts.

We also have the capacity to use small animal immuno-positron emission tomography (iPET). IPET is a new, sophisticated imaging system that can facilitate our understanding of the NHL-targeting of these new drugs, and to rapidly enhance new drug development. Therefore, our Specific Aims are to:

II. Body

Progress within the first 2 years of the granting period included identification and initial characterization of several CD22-binding peptides which has been summarized in the year 1 and 2 progress reports.

Below the research accomplishments for year three will be summarized and organized based on the proposed aims and goals as outlined in the Statement of Work (SOW).

Aim I is to *identify and characterize CD22-binding peptides that initiate signal transduction and results in apoptosis. CD22 binding and internalization will be optimized to enhance the highly specific and effective lymphomacidal properties demonstrated by the parent mAbs.*

The goals of Aim I are:

1. *To design and synthesize peptides derived from the highly conserved CDRs of anti-CD22 ligand blocking mAbs and characterize their binding in vitro to B-cell NHL lines and normal tonsilar B-cells. –* ***Completed year 1***

2. *The physiologic effects of high affinity peptides: initiation of signal transduction, and effects on cell growth and apoptosis, will be studied.-****Completed year 2***

3. *High affinity binding peptides will be further characterized by N and C-terminal deletion analysis and alanine walk analysis to identify the crucial amino acids for molecular recognition. Mutational analysis will be done to identify more peptides with enhanced affinity. -****Completed year 1***

4. *Promising peptides that initiate signal transduction and mediate apoptosis will be further assessed in vivo for their lymphomacidal properties using a nude mouse xenograft model.*

As proposed and in an attempt to identify peptides with higher affinity and better apoptotic and lymphomacidal activity we used the one bead, one peptide combinatorial library developed by Dr. Kit Lam. Previous studies with these CD22-binding peptides demonstrated that amino acid number 9 and 21 could be changed to alanine without consequence to CD22 binding. Thus these were strategic sites for modification by the peptide library to potentially improve affinity and killing. A specific library was created for each specific aminio acid site (Aa9 and Aa21) as previously described and proposed.
The library screening involved incubation of the peptide-coated beads with the CD22-positive cell line Ramos
(figure 1).

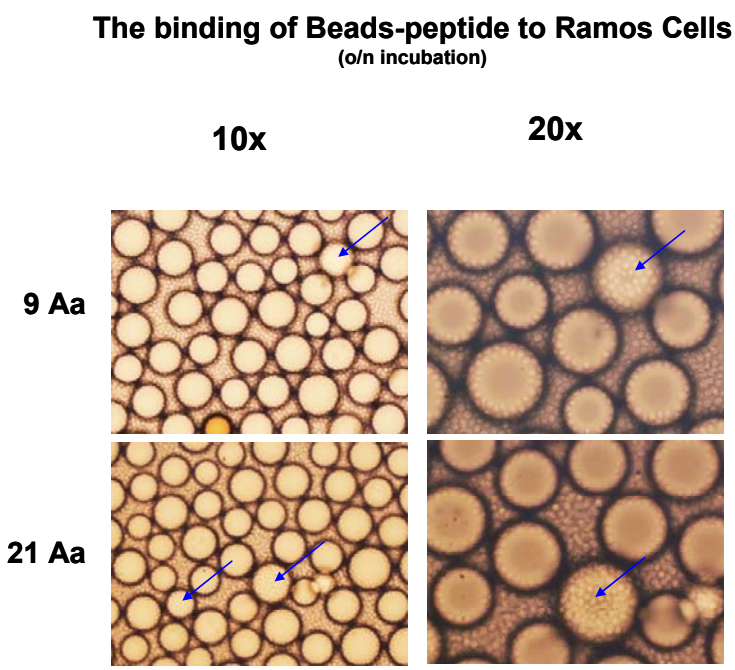

Figure 1. Ramos B cells were incubated with the site-specific peptide library and beads that bound to Ramos cells are indicated (blue arrows) and were manually selected for further analysis.

These peptides were identified and produced in larger quantities to examine their cytotoxic potential. This was done by incubating with Ramos cells for 3 days and assessing cell viability (figure 2).

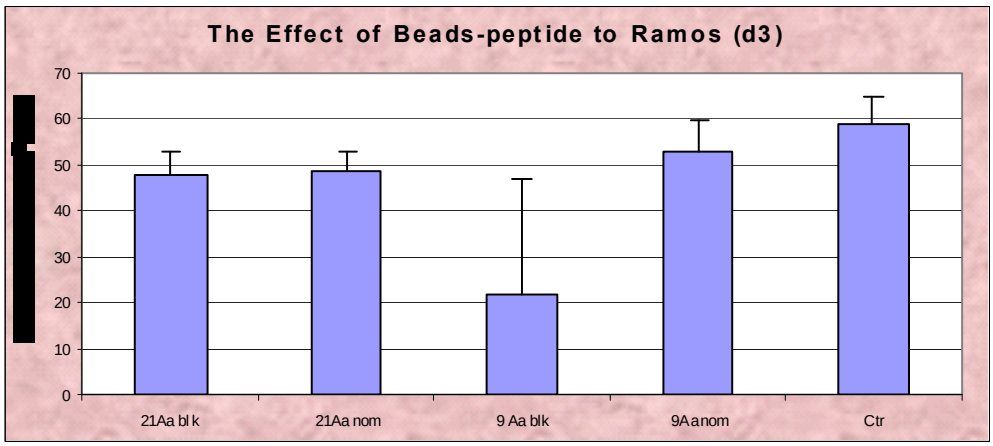

Figure 2. Aa 9 and Aa21 peptide cell killing assay. 20ug/cc of each peptide was incubated with Ramos cells for 3 days and assessed for viability using trypan blue exclusion. The results represent the average of 3 replicate experiments, with the error bars representing the standard deviation (SD).

These results suggest that peptide Aa9 has significant killing potential although the SD was relatively high. These studies are currently being repeated with higher peptide doses and several CD22 positive NHL cell lines.

We next wanted to assess the potential of peptide 9Aa to kill a number different CD22 positive cell lines, (figure 3).

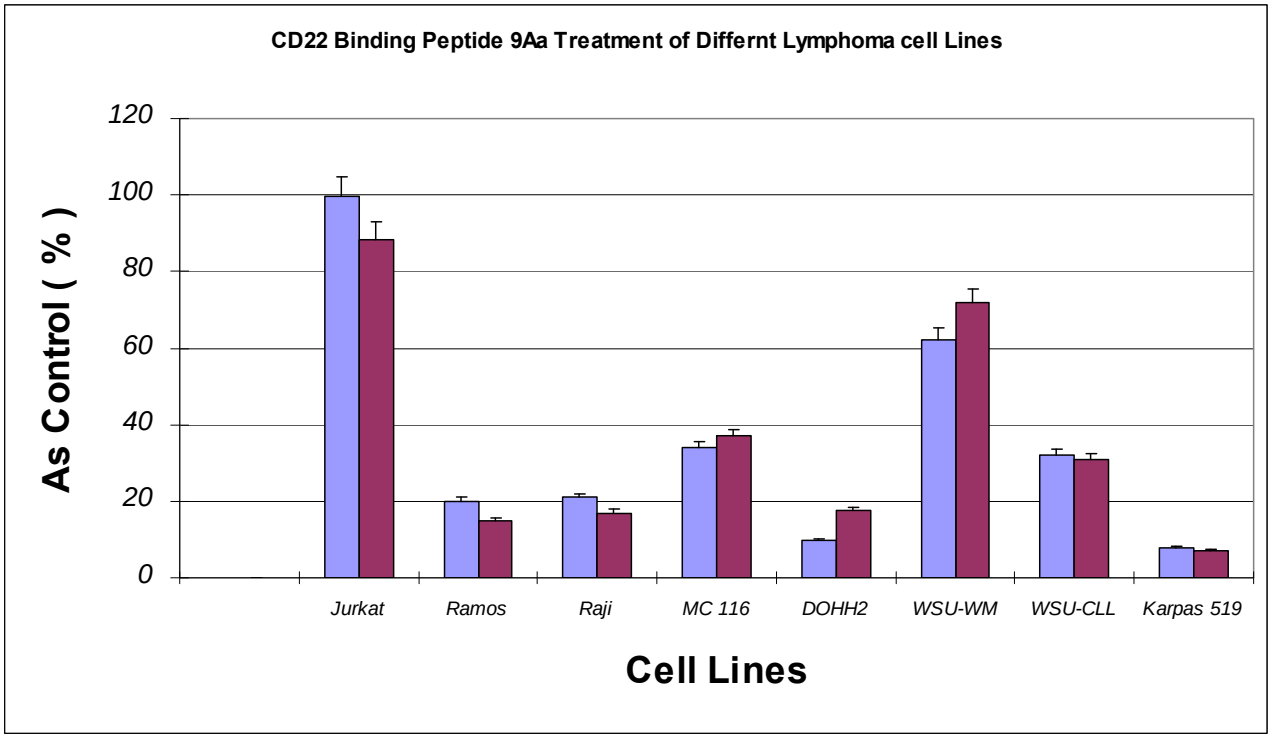

Figure 3. Cytotoxicity assay of peptide Aa9 in several CD22 + NHL cell lines. Cells were incubated with peptide Aa9 (20ug/cc) for 3 days and assessed for viability by trypan blue exclusion. The data presented represents the average of 3 replicate experiments with the error bars representing the SD.

This data verified the cytotoxic activity of 9Aa modification of peptide 5. This lack of significant killing of the CD22 negative cell line, Jurkat, suggested tissue (B cell) specificity. However we next looked at the tissue specificity of the Aa9 peptide in several CD22 negative cell lines. We found that when we examined binding to a number of solid tumor cell lines (including breast, lung and prostate) peptide Aa9 also bound to these cell lines, suggesting that is was less specific. We then compared the binding of peptide Aa9 to peptide 5 and found that while it may have killed NHL cell lines more effectively it was less NHL-specific. We then consulted with our collaborator Dr. Kit Lam and we agreed that more NHL-, and CD22-specific peptides were needed. We decided to develop a CD22 specific cell line to better select CD22-binding peptides. We did this first by subcloning CD22-encoding cDNA (obtained from Dr. John Kehrl NIAID, NIH) into the pCDNA eukaryotic expression vector. After sequence verification this vector was transfected into the CD22 negative cell line 293T cells. The transfected cells were then selected

by G418 resistance. Subcloned cell lines with high levels of CD22 surface expression were selected by CD22-directed FACS-based cell sorting. This process was recently completed and peptide 5 library modified at Aa 9 and Aa21 will be used to select peptides that are CD22-specific and with greater affinity. Having CD22 positive and negative cell lines will allow for identification of peptides within the library that will spefically bind to CD22. Nonspecific binding peptides will be eliminated but removing those that bind to the untransfected 293 cells. Again this will potentially allow for selection modifications to peptide 5 that specifically bind CD22 with greater affinity and more specificity.

Aim II *is to optimize CD22-mediated signal transduction and the lymphomacidal properties of the ligand blocking anti-CD22 mAbs and peptides with CD22-specific phosphatase inhibition.*

Goals for Aim II are*:*

*1. To analyze CD22-mediated signal transduction and apoptosis manipulated by tyrosine phosphatase inhibition in vitro.-**Completed for peptide 5, pending for CD22-binding peptides under development.***

2. To assess the efficacy of combining phosphatase inhibitor(s) with the anti-CD22 ligand blocking mAb and peptides in human NHL xenograft models.-Pending selection and analysis of recently developed peptides

III. Annual Report Summary/Key Research Accomplishments

Years 1 and 2

- Peptides 5 and 44 were found to activate the SAPK and p38 signal transduction pathways.
- Based on the mutational analysis of CD22 binding peptide 5 several additional peptides were identified that effectively kill lymphoma cells.
- These peptides (#40, 41, and 44) were shown to preferentially kill B cells, and their cytotoxic effects were dose responsive.
- The cytotoxic effects of peptide 41 was active in several NHL cell lines that represent diverse NHL subtypes.
- Peptides 40, 41, and 44 killed normal as well as malignant B cells.
- Peptide 41 induced apoptosis in malignant B cells approximately to same degree as peptide 5 and considerably better than Rituxan.
- We found that peptide 41 partially blocked binding of the anti-CD22 mAb HB22.7 and thus likely binds to the same CD22 epitope and only binds transiently.
- We demonstrated that peptide 41 mediated the production of pro-apoptotic soluble factors.
- We demonstrated that phosphatase inhibition augmented the cytotoxic potential of peptide 5.

Year 3

- Used the combinatorial peptide library to identify modified peptide 5 that bound to multiple B cell NHL cell lines.
- Identified peptide 5 derivative Aa9 that had significant apoptotic potential in a number of NHL cell lines
- Found that modified peptide 5, Aa9 had less tissue specificity

- Developed a CD22 transfected cell line that has a high level of CD22 surface expression that is currently being used to screen the combinatorial library for more specific CD22-binding peptides

IV. Reportable Outcomes

Currently there are no additional publications. The data presented above is reportable but will only be published when verified and additional data has been generated that will facilitate publication. All subsequent publications will acknowledge the DOD Investigator-Initiated Research Award Number (W81XWH-07-1-0471).

V. Conclusion

The studies presented herein demonstrate that a peptide derived from CDR2 of the anti-CD22 mAb HB22.7 (Peptide 5) binds to CD22 on B lymphocytes, mediates internalization, signal transduction, and killing of lymphoma cells. We also demonstrated that this peptide can be used as a vehicle to deliver pro-apoptotic payload to lymphoma cell cells that enhance the killing potential of the parent mAb and peptide (work completed in year 1). Studies completed in year 2 identified additional peptides (#40, 41, and 44) that were developed from the mutational analysis of peptide 5 that have been found to be even more effective at killing lymphoma cells and inducing apoptosis. Interestingly these new peptides appear to mediate their cytotoxic effects by inducing malignant B cells to produce pro-apoptotic soluble factors.

A considerable amount of time was spent using the focused combinatorial peptide library system to develop modifications of peptide 5 that had higher affinity and greater apoptotic potential. While this resulted in identification of peptides (Aa9) that had significant apoptotic potential in multiple NHL cell lines, they had less tissue specificity making them unsuitable for further development. Subsequent studies focused on the development of CD22 specific cell lines that would allow for better selection of CD22-binding peptides that are more specific. The development of these cell lines has been completed and further peptide selection using the combinatorial library are ongoing. Once these peptides are optimized the final goals of this proposal will be completed, including *in viv*o NHL xenograft studies and immuno-PET scanning.

VI. Literature Citations

1. Sato, S., Tuscano, J. M., Inaoki, M., and Tedder, T. F. (1998) *Semin Immuno* **10**(4)**,** 287-97.
2. Tuscano, J., Engel, P., Tedder, T. F., and Kehrl, J. H. (1996) *Blood* **87**(11)**,** 4723-30.
3. Tuscano, J. M., Riva, A., Toscano, S. N., Tedder, T. F., and Kehrl, J. H. (1999) *Blood* **94**(4)**,** 1382-92.
4. Tuscano, TM, O'Donnell RT, Miers LA, Kroger LA, Kukis DL, Lamborn KR, Tedder TF and DeNardo DL. *Blood* 101(9), 3641-3647, 2003.
5. Law, C. L., Aruffo, A., Chandran, K. A., Doty, R. T., and Clark, E. A. (1995) Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. *J Immunol* 155(7), 3368-76
6. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Treatment of non-Hodgkin's lymphoma xenografts with the HB22.7 anti-CD22 monoclonal antibody and phosphatase inhibitors improves efficacy. *Cancer Immunol Immunother.* 2009 Mar 18
7. Pearson D., O'Donnell RO, Cerejo M, McKnight HC, Wang X, Lam KS, and Joseph M. Tuscano. CD22-Binding Peptides Derived from Anti-CD22 Ligand Blocking Antibodies

Retain the Targeting and Cell Killing Properties of the Parent Antibodies and May Serve as a Drug Delivery Vehicle. Int J Pept Res Ther (2008) 14:237–246
8. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Treatment of non-Hodgkin's lymphoma xenografts with the HB22.7 anti-CD22 monoclonal antibody and phosphatase inhibitors improves efficacy. *Cancer Immunol Immunother.* 2009 Mar 18.
9. O'Donnell RT, Pearson D, McKnight HC, Ma YP, Tuscano JM. Phosphatase inhibition augments anti-CD22-mediated signaling and cytotoxicity in non-hodgkin's lymphoma cells. *Leuk Res.* 2009 Feb 21.